AD_____

Award Number: W81XWH-10-1-0195

TITLE: Beta Catenin in Prostate Cancer Apoptosis

PRINCIPAL INVESTIGATOR: Basabi Rana, Ph.D.

CONTRACTING ORGANIZATION: Loyola University
                          Maywood, IL-60153

REPORT DATE: July 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and
should not be construed as an official Department of the Army position, policy or decision
unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-12-2014	Final	

4. TITLE AND SUBTITLE
Beta Catenin in Prostate Cancer Apoptosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Basabi Rana, Ph.D.

E-Mail: brana@luc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Loyola University

Maywood, IL-60153-3328

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT During this funding period, we performed TRAIL-TZD-mediated apoptotic studies in androgen dependent (LNCaP and 22RV1) and androgen independent (DU145 and PC3) prostate cancer cells and the response was maximal in the LNCaP cells with $50\mu M$ TZD and 100ng/ml TRAIL combination. This apoptotic response is also associated with increased β-catenin cleavage, indicating its potential role. Knockdown of β-catenin expression and overexpression of β-catenin modulated TRAIL-TZD-induced apoptosis. Studies with the cleaved β-catenin protein revealed that this cleaved fragment losses interaction with TCF4, while retaining strong interaction with E-cadherin and α-catenin. These indicated (i) a potential mechanism by which TRAIL-TZD antagonizes β-catenin/TCF4-induced transcription and (ii) suggested that E-cadherin and α-catenin interaction might be critical for apoptosis induction. Based on Mass Spec data various myc β-catenin (D/A) mutants were created but they were unable to significantly antagonize β-catenin cleavage and apoptosis. Studies also showed that GSK3β inhibition promotes TRAIL-induced apoptosis and TRAIL-TZD significantly reduces expression of GSK3β and α and increases pGSK3β^{Ser9} levels. In addition, this apoptosis pathway seems to involve AMPK, since AMPK-dominant negative (DN) overexpression significantly attenuates TRAIL-TZD-induced apoptosis and β-catenin cleavage. The C42 and C42B cells showed significant apoptosis with TRAIL-TZD *in vitro* and C42 cells formed *in vivo* xenografts in nude mice. Treatment with TRAIL-TZD *in vivo* however, didn't show any significant effect on prostate tumor regression.

15. SUBJECT TERMS
TRAIL, Troglitazone, beta-catenin, GSK3beta, apoptosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			
U	U	U	UU	36	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction:

During the funding period, we have worked towards completing all the 3 specific aims as was listed in the approved Statement of Work. However, there were some setbacks in the overall studies, since two personnel have left the program in previous funding period. Since then new personnel has been hired and after an initial delay (covering the training period) we have made significant progress in accomplishing the goals listed in the project.

Background:

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death in males (1), (2). Surgical resection and hormonal therapy (with anti-androgens) are the two major forms of treatment currently available, which is not effective at a late stage (hormone independent forms) of the disease. Designing efficient therapeutic agents that can target both hormone dependent and independent forms of the cancer are thus critically important. Since most of the anticancer therapies limit tumor growth via inducing apoptosis, identification of a novel target for drug induced apoptosis will be helpful for treating resistant forms. One such target is β-catenin, a downstream mediator of Wnt pathway and known to be closely linked with tumorigenesis (3). Overexpression of β-catenin can induce early events of prostate tumorigenesis (4), (5), and contribute to prostate cancer cell growth (6). Activating mutations of β-catenin have also been reported in approximately 5% of human prostate cancers (7), (8). β-catenin can also augment transcriptional function of androgen receptor (AR) (9), (10) (11). Since β-catenin can promote survival via increasing expression of survival related genes (12), (13), decreasing β-catenin expression might be a critical event in activating the apoptotic pathway.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor super-family of death-inducing ligands, has gained increased attention due to its unique capability of inducing apoptotic cell death specifically in cancer cells, without any significant toxicity towards normal cells (14). TRAIL receptors are expressed in both androgen dependent and independent prostate cancer cells, although, some of these cells develop TRAIL resistance (15). Studies by others have revealed that combinatorial treatment with TRAIL and ligands of PPARγ (Peroxisome Proliferator Activated Receptor gamma) can ameliorate TRAIL resistance and induce apoptosis in TRAIL resistant breast cancer cells (16). Despite this information, the detailed mechanism how this drug combination promotes TRAIL sensitivity is still unknown. Identification of downstream molecules regulating this apoptotic pathway is critically required not only to overcome TRAIL resistance, but also to understand the detailed mechanism involved, which can be utilized towards future drug designing. Since TRAIL receptors are expressed in all prostate cancer cells, this therapy might be effective in targeting prostate cancers irrespective of their androgen status.

In our preliminary data, that supported funding of this grant, co-treatment of TRAIL resistant prostate (LNCaP) and liver (Huh-7) cancer cells with a combination of TRAIL and PPARγ ligand Troglitazone (TZD) reduced TRAIL resistance and significantly increased their apoptotic potential. Interestingly, this apoptosis was also associated with a dramatic reduction in the expression of β-catenin protein and a cleavage of β-catenin preceding combinatorial drug-induced apoptosis. Regulation of β-catenin seemed to be independent of the conventional GSK3β-mediated pathway and involved caspase activation. Based on these, in the current application we proposed to study in detail the role of β-catenin and GSK3β in drug-induced

apoptosis of prostate cancer cells (*in vitro*) and prostate cancer xenografts (*in vivo*). The specific aims included: *1) To determine the role of β-catenin in drug-induced apoptosis of prostate cancer cell lines, 2) To determine the role of GSK3β in potentiating drug-induced β-catenin cleavage and apoptosis and 3) Whether β-catenin mediates drug-induced apoptosis in prostate xenografts in vivo.*

Body:
The research accomplishments for the entire funding period along with each task included in the approved Statement of Work are outlined below:
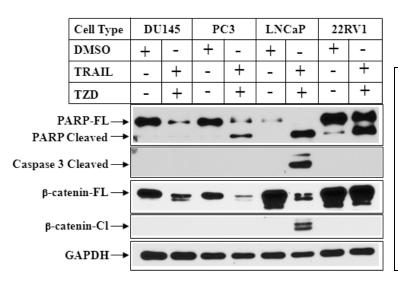
Task 1: To determine the role of β-catenin in drug-induced apoptosis of prostate cancer cell lines (1-36 months)
Subaims 1(i)-1(ii):
The results described in the preliminary studies section of this grant proposal were obtained initially utilizing LNCaP prostate cancer cells and Huh-7 hepatocellular carcinoma cells (HCC). After the funding of the grant, extensive studies were designed with TRAIL and Troglitazone (TZD) combination in various prostate cancer cells to determine whether this pathway operates in other prostate cancer cells and is a generalized event. In addition, since LNCaP cells (which respond to this combination treatment with potent apoptosis) are androgen sensitive cells, we also utilized androgen insensitive cell types to determine whether this apoptosis pathway is only specific to androgen sensitive cell types. In order to determine the role of β-catenin in mediating this apoptotic response, the correlation of apoptosis with β-catenin expression and cleavage were also determined. This information is critically important clinically to define which prostate cancer types might respond to this form of therapy. The following results were obtained:

Effect of TRAIL-TZD combination on the apoptosis potential and β-catenin expression of androgen sensitive and androgen insensitive prostate cancer cells:
To address this questions we utilized two different cell types that are androgen sensitive (LNCaP and 22RV1) and two that are androgen insensitive (PC3 and DU145). These cells were plated at high density followed by treatment with TRAIL and TZD for 24 hours. Extensive analysis of all these cells showed that the LNCaP and 22RV1 produced m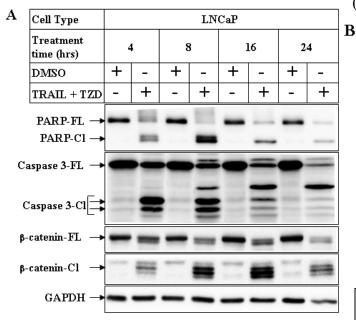aximal response to this combination treatment with a significant increase in PARP cleavage, indicating increased apoptosis (Fig 1). These results also
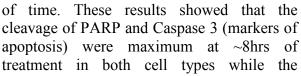
Fig 1: Effect of TRAIL-TZD on prostate cancer cell apoptosis: Androgen sensitive (LNCaP and 22RV1) and androgen insensitive (DU145 and PC3) prostate cancer cells were treated with either DMSO or a combination of 100ng/ml TRAIL and 50μM TZD for 24 hours. Cells were then harvested and equal amount of total cell lysates were analyzed by Western Blots utilizing various antibodies. FL-indicates Full length and Cl – indicates cleaved.

Cell Type	DU145		PC3		LNCaP		22RV1	
DMSO	+	-	+	-	+	-	+	-
TRAIL	-	+	-	+	-	+	-	+
TZD	-	+	-	+	-	+	-	+

indicated a distinct cleavage of β-catenin protein in the LNCaP cells when treated with the drug combination (Fig 1, see β-catenin-cl panel). This cleaved form was absent in all other cell types and was similar to the cleaved caspase 3 expression as shown in Fig 1, suggesting strongly that this cleaved β-catenin expression correlates very well with the apoptotic potential of the prostate cancer cells. Treatment with this drug combination, however, produced a substantial decrease in the expression of the full length protein in almost all the cell types tested (see β-catenin-FL panel). These indicated that the decrease in β-catenin full length protein and β-catenin cleavage operate via two independent pathways with the latter being correlated with apoptosis. It will however, be interesting to determine whether the decrease in full length β-catenin protein is a prerequisite for the induction of apoptosis. In an earlier report, we have demonstrated that PPARγ activation via TZD can lead to a reduction of full length β-catenin expression, which operates via a GSK3β-independent non-conventional pathway (17). It is thus conceivable that this reduction in the full length β-catenin protein expression might be due to the effects of TZD in the drug combination.

Time course of apoptosis and β-catenin cleavage following TRAIL-TZD stimulation in prostate cancer cells: In order to determine the peak-time of apoptosis following treatment with TRAIL and TZD combination, both LNCaP and DU145 cells were treated with a combination of TRAIL (100ng/ml) and TZD (50μM) for various lengths
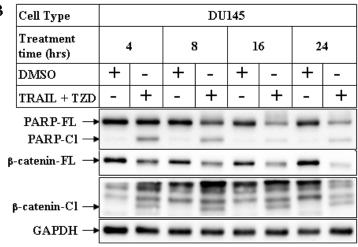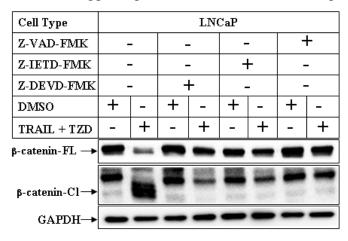

A LNCaP

Cell Type	LNCaP							
Treatment time (hrs)	4		8		16		24	
DMSO	+	–	+	–	+	–	+	–
TRAIL + TZD	–	+	–	+	–	+	–	+

PARP-FL →
PARP-Cl →
Caspase 3-FL →
Caspase 3-Cl →
β-catenin-FL →
β-catenin-Cl →
GAPDH →

B DU145

Cell Type	DU145							
Treatment time (hrs)	4		8		16		24	
DMSO	+	–	+	–	+	–	+	–
TRAIL + TZD	–	+	–	+	–	+	–	+

PARP-FL →
PARP-Cl →
β-catenin-FL →
β-catenin-Cl →
GAPDH →

Fig 2: Time course of TRAIL-TZD-induced apoptosis and β-catenin cleavage in prostate cancer cells. LNCaP (**A**) and DU145 (**B**) cells were treated with either DMSO or a combination of TRAIL (100ng/ml) and TZD (50μM) for the indicated time points, followed by Western Blot analyses.

of time. These results showed that the cleavage of PARP and Caspase 3 (markers of apoptosis) were maximum at ~8hrs of treatment in both cell types while the cleavage of β-catenin showed a maximum effect at 16hrs (Fig 2A and B). In addition two other prostate cancer cells (C42 and C42B) were also tested for their responsiveness to TRAIL-TZD combination, which were highly sensitive (see Task 3 results).

TRAIL-TZD-induced cleavage of β-catenin is mediated via activation of Caspase 3 and 8: In order to determine whether Caspases were involved in mediating β-catenin cleavage following treatment with a combination of TRAIL and TZD, LNCaP cells were pretreated separately with inhibitors of either Caspase 3 (Z-DEVD-FMK), or Caspase 8 (Z-IETD-FMK) or pan Caspase inhibitor (Z-VAD-FMK) prior to TRAIL-TZD treatment. These results shown in Fig 3 showed that cleavage of β-catenin was completely antagonized when pretreated with the Caspase inhibitors, suggesting an involvement of both Caspase 3 and 8 in mediating this. However, the reduction of full length β-catenin observed with TRAIL-TZD was fully

Cell Type	LNCaP							
Z-VAD-FMK	-		-		-		+	
Z-IETD-FMK	-		-		+		-	
Z-DEVD-FMK	-		+		-		-	
DMSO	+	-	+	-	+	-	+	-
TRAIL + TZD	-	+	-	+	-	+	-	+

β-catenin-FL →

β-catenin-Cl →

GAPDH →

Fig 3: Effect of Caspase inhibitors on TRAIL-TZD-induced modulation of β-catenin: LNCaP cells were treated with TRAIL-TZD for 16hrs following a pretreatment for 1hour with none (lanes 1 & 2), or 25µM Z-DEVD-FMK (lanes 3 & 4), or 25µM Z-IETD-FMK (lanes 5 & 6) or 50µM Z-VAD-FMK (lanes 7 & 8). Western Blot analyses were performed next with the antibodies indicated.
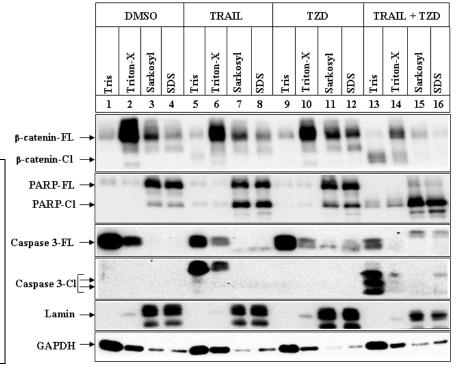
antagonized when pretreated with pan caspase inhibitor and not with those of caspase 3 and 8. These suggested that TRAIL-TZD-induced reduction of FL-β-catenin might be mediated via Caspases other than 3 and 8.

Effect of TRAIL-TZD on the localization of β-catenin: β-catenin can be localized in various compartments of the cell, which 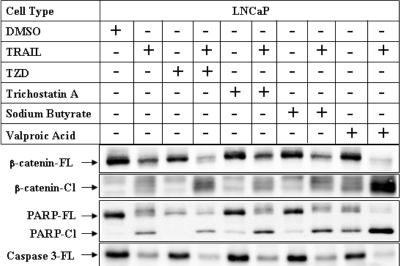include membrane as well as cytoplasm and nuclear fractions. To determine whether TRAIL-TZD-cleaved β-catenin was enriched in any particular cellular compartment, LNCaP cells were treated with TRAIL and TZD alone or in combination for 16hrs and extracted sequentially as

Fig 4: Localization of cleaved β-catenin during TRAIL-TZD-induced apoptosis: LNCaP cells were treated with DMSO or TRAIL and TZD alone or on combination for 16hrs followed by sequential extraction with 50mM Tris-HCl, 1% Triton-X-100, 1% Sakosyl and 2% SDS. Western Blot analyses were performed with the antibodies indicated.

	DMSO				TRAIL				TZD				TRAIL + TZD			
	Tris	Triton-X	Sarkosyl	SDS	Tris	Triton-X	Sarkosyl	SDS	Tris	Triton-X	Sarkosyl	SDS	Tris	Triton-X	Sarkosyl	SDS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

β-catenin-FL →
β-catenin-Cl →

PARP-FL →
PARP-Cl →

Caspase 3-FL →

Caspase 3-Cl →

Lamin →

GAPDH →

described earlier (18). The results shown in Fig 4 indicated that β-catenin FL was normally enriched in the membrane and extracted in Triton-X containing fraction (lane 2), which was gradually diminished with TRAIL and TZD treatments (lanes 6, 10, 14). The cleaved β-catenin fragment, however, was exclusively localized in the soluble compartment and extracted in the Tris fraction (lane 13). This was also true for cleaved Caspase 3, which showed an exclusive localization in the soluble compartment (lane 13). Cleaved PARP, however, was enriched in the nuclear/cytoskeletal compartment and was extracted in Sarkosyl and SDS fractions (lanes 15, 16). Caspase 3 FL was also enriched in the soluble fraction (lane 1), suggesting the possibility that targeting β-catenin towards this fraction might increase the accessibility of β-catenin for caspase 3-induced cleavage.

Effect of HDAC inhibitors on TRAIL-induced apoptosis and β-catenin cleavage in prostate cancer cells: In order to determine, whether apoptosis induction via HDAC inhibitors also produces a similar cleavage of β-catenin, LNCaP cells were treated with TRAIL in combination with either TZD or various HDAC inhibitors. These studies (Fig 5) revealed that combination treatment of TRAIL with each of the HDAC inhibitors induced apoptosis to varying extent and was associated with a corresponding cleavage of β-catenin, confirming again that apoptosis-related Caspase activation is associated with β-catenin cleavage. Combination treatment with TRAIL and Valproic acid however showed the maximum response towards both apoptosis induction and β-catenin cleavage (lane 10).

Cell Type	LNCaP									
DMSO	+	-	-	-	-	-	-	-	-	-
TRAIL	-	+	-	+	-	+	-	+	-	+
TZD	-	-	+	+	-	-	-	-	-	-
Trichostatin A	-	-	-	-	+	+	-	-	-	-
Sodium Butyrate	-	-	-	-	-	-	+	+	-	-
Valproic Acid	-	-	-	-	-	-	-	-	+	+

Fig 5: Effect of HDAC inhibitors on TRAIL-induced apoptosis and β-catenin cleavage: LNCaP cells were treated for 16hours with TRAIL and TZD alone or in combination (lanes 2-4). These cells were also treated with various HDAC inhibitors alone or in combination with TRAIL; 500ng/ml Trichostatin A (lanes 5 & 6), 5mM Sodium Butyrate (lanes 7 & 8) or 5mM Valproic acid (lanes 9 & 10). Western analyses were performed next with the antibodies indicated.

Effect of TRAIL-TZD on the interaction of β-catenin with its binding partners: To determine whether the cleaved β-catenin fragment still retains its interaction with its binding partners E-cadherin and TCF4, Immunoprecipitation and Immunoblotting combination studies were designed in two different prostate cancer cell lines (LNCaP and 22RV1) following treatment with TRAIL and TZD. These studies showed a distinct interaction of both full length and cleaved β-catenin with E-cadherin and α-catenin (Fig 6A, 6B), whereas this cleaved fragment was unable

to interact with TCF4 (Fig 6A). These interesting results indicate that; (i) intact β-catenin-E-cadherin-α-catenin interaction might be needed for optimal β-catenin cleavage and (ii) TRAIL-TZD treatment might be completely antagonizing β-catenin/TCF-mediated transcriptional activity via abolishing β-catenin's interaction with TCF4.
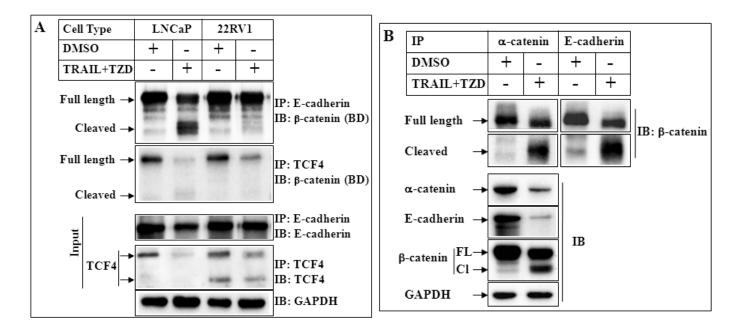

Fig 6: Effect of TRAIL-TZD on β-catenin's interaction with E-cadherin, TCF4 and α-catenin: (A) LNCaP and 22RV1 prostate cancer cells were treated with DMSO or a combination of TRAIL and TZD, as in Fig 1. Equal amounts of lysate were then Immunoprecipitated with either E-cadherin antibody (top panel) or with TCF4 antibody (second panel). Each of these Immunoprecipitates was then Immunoblotted separately with an antibody against β-catenin. **(B)** LNCaP prostate cancer cells were treated with DMSO or TRAIL -TZD for 16hrs. Equal amounts of lysates were then Immunoprecipitated with either E-cadherin antibody or α-catenin antibody (top panels). Each of these Immunoprecipitates was Immunoblotted separately with an antibody against β-catenin to detect Full length (FL) or cleaved (Cl) β-catenin. The bottom panels represent Western Blots of the lysates with the corresponding antibodies.

Subaim 1(iii), (v), (vi):
Based on our earlier antibody mapping data and in order to map the caspase cleavage site on β-catenin protein, we initially focused on myc-tagged-β-catenin-FL (1-781aa) and the deleted, myc-β-catenin-ΔN (131-781aa) deletion mutant to create cleavage-resistant (D/A) mutants. These included D583A, D624A, D583A/D624A, D751A, D764A, D751A/D764A and were created with both myc-β-catenin–FL (1-781) and myc-β-catenin-ΔN (131-781aa). Even though we initially hypothesized that the deleted β-catenin-ΔN (131-781aa) construct will be better in identifying the cleavage site, we were unable to detect any cleaved fragment with β-catenin antibody when transfected in the LNCaP cells. We thus decided to use β-catenin-FL (1-781aa) and its D/A mutants for the future mapping studies.

Effect of overexpression of β-catenin mutants on TRAIL-TZD-induced apoptosis and β-catenin cleavage: In order to determine the effect of β-catenin D583A and D624A mutations on β-catenin cleavage and TRAIL-TZD-induced apoptosis, these mutants were transiently transfected in LNCaP cells followed by treatment. The results shown in Fig 7A indicated that these constructs expressed β-catenin protein at the correct sizes (shown by the myc blot). In addition, the D583A mutation seemed to partially inhibit both endogenous β-catenin and PARP cleavage. These suggested that D583A might an important site for β-catenin cleavage by TRAIL-TZD pathway, although additional sites might also be present. In an additional experiment, various D/A constructs created (total 6) were transfected separately in LNCaP cells followed by TRAIL-TZD treatment. This also showed that all the mutants expressed β-catenin protein at the correct sizes (Fig 7B, myc blot). However, due to variability in transfection, we were unable to determine conclusively whether D583A or any of the other D/A mutants was resistant to TRAIL-TZD-induced apoptosis.
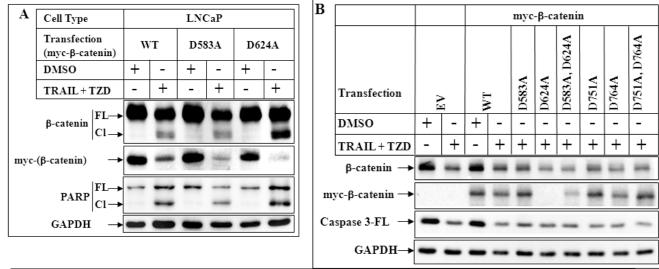

Fig 7: Effect of overexpression of β-catenin (WT) and D/A mutants in LNCaP cells: (A) LNCaP cells were transiently transfected with β-catenin-WT, D583A or D624A mutant constructs and treated with TRAIL-TZD for 16hours. Equal amounts of protein from each sample were then analyzed by Western Blots. **(B)** LNCaP cells were transiently transfected with Empty vector (EV, lanes 1&2), β-catenin-WT (lanes 3&4), or various D/A mutant constructs (lanes 5-10) and treated with TRAIL-TZD for 16hours. Equal amounts of protein from each sample were then analyzed by Western Blots.

To conclusively identify these cleavage sites, we also analyzed the cleaved product to identify the specific cleavage site using Mass Spectrometry. The initial step of Mass Spectrometry is to obtain a Coomassie-stainable band following Immunoprecipitation of the specific the protein (cleaved β-catenin in this case). There were several obstacles in this initial Immunoprecipitation stage, since we were unable to identify any suitable commercially available β-catenin antibody that can successfully Immunoprecipitate the cleaved β-catenin fragment. As shown in Figs 6A&B, since the cleaved fragment retains interaction with E-Cadherin, we also tried to Immunoprecipitate this with E-cadherin antibody initially, but were unable to enrich this to a Coomassie-stainable band that is a prerequisite for Mass Spec analysis. After several attempts with significant enrichment of the lysates containing the cleaved β-catenin fragment, we were

successful in detecting the cleaved β-catenin fragment in E-Cadherin IP after Coomassie stain (Fig 8). The cleaved fragment detected at ~70KD (red box) was then sent out for Mass Spectrometric analysis using Bottom up detection system. This detected 3 major cleaved products corresponding to D32, D583 and D764 of beta-catenin. We have already created the D583A and D764A mutants earlier and later also created the D32A single mutant. However, these mutants were unable to significantly block β-catenin cleavage or apoptosis.
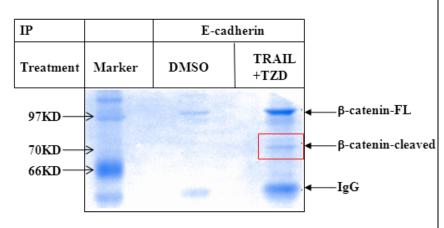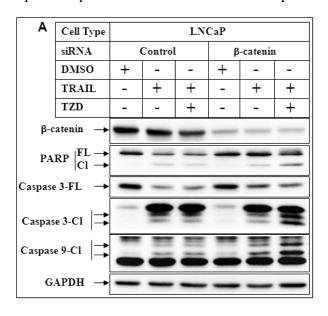

Fig 8: Detection of cleaved β-catenin fragment following Coomassie stain: LNCaP cells were treated with DMSO or TRAIL-TZD as in Fig 7, followed by Immunoprecipitation with E-cadherin antibody. The Immunoprecipitates were resolved on SDS-PAGE followed by Coomassie Blue staining. Lane 1 contains protein molecular weight marker. The cleaved β-catenin band at ~70 KD (red box) was sent out for Mass Spec analysis.

Subaim 1(iv):

Role of β-catenin on TRAIL or TRAIL-TZD-induced apoptosis in LNCaP prostate cancer cells:
Effect of knockdown: To understand the role of β-catenin in mediating apoptosis and survival in AR-positive prostate cancer cells more in depth and to see which caspases are affected,
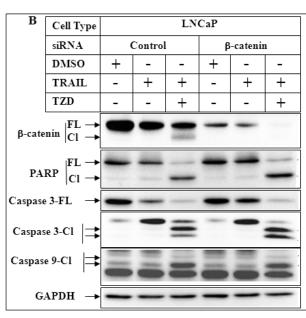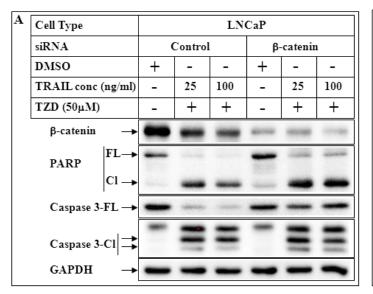

Fig 9: Effect of β-catenin knockdown on TRAIL or TRAIL-TZD-induced apoptosis: LNCaP cells were transiently transfected with control or β-catenin siRNA for 72hrs followed by TRAIL-TZD treatment for 8hrs **(A)** or 16hrs **(B)**. The samples were analyzed by Western Blots with the antibodies indicated.

β-catenin-siRNA studies were performed in LNCaP cells. These results showed a significant reduction in endogenous β-catenin expression with β-catenin-siRNA (Fig 9 A&B). TRAIL-TZD studies were performed for 8 hrs and 16hrs to see any time-dependent effects. These results showed that knockdown of endogenous β-catenin rather increased the cleavage of caspase 3 and 9 as well as PARP. In addition, the effect of β-catenin-siRNA was also determined on increasing concentration of TRAIL in TRAIL-TZD combination. Knockdown of β-catenin expression induced more apoptosis with both 25ng/ml and 100ng/ml TRAIL combination (Figs 10 A and B). These studies suggested a prosurvival role of this protein in AR-positive LNCaP cells.
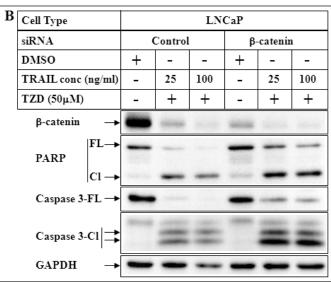

A Cell Type	LNCaP					
siRNA	Control			β-catenin		
DMSO	+	-	-	+	-	-
TRAIL conc (ng/ml)	-	25	100	-	25	100
TZD (50μM)	-	+	+	-	+	+

B Cell Type	LNCaP					
siRNA	Control			β-catenin		
DMSO	+	-	-	+	-	-
TRAIL conc (ng/ml)	-	25	100	-	25	100
TZD (50μM)	-	+	+	-	+	+

Fig 10: Effect of β-catenin knockdown on TRAIL (concentration) and TZD-induced apoptosis: LNCaP cells were transiently transfected with control or β-catenin siRNA as in Fig 9 followed by TRAIL-TZD treatment for 8hrs **(A)** or 16hrs **(B)**. The samples were analyzed by Western Blots.

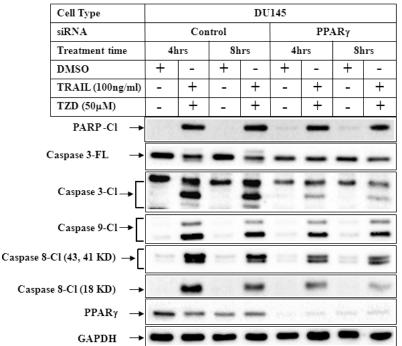| Cell density	0.4 x 10⁶				0.6 x 10⁶			
Transfection	EV		myc-βcat WT		EV		myc-βcat WT	
DMSO	+	-	+	-	+	-	+	-
TRAIL+TZD	-	+	-	+	-	+	-	+

Effect of overexpression: To conclusively establish a prosurvival function of β-catenin in the AR-positive prostate cancer cells, the LNCaP cells were transiently transfected with either empty vector (EV) or β-catenin (WT) vector followed by TRAIL-TZD treatment for 16hrs. Since this apoptosis pathway is dependent on cell density (data not shown), the cells in this experiment were plated at two different densities. The results (Fig 11) indicated that at both cell densities, overexpression of β-catenin attenuated TRAIL-TZD-induced PARP cleavage, indicating again that β-catenin mediates a pro-survival pathway in these cells. The effects of overexpression were modest likely due to the low transfection efficiency of LNCaP cells.

Fig 11: Effect of β-catenin overexpression on PARP cleavage: Subconfluent populations of LNCaP cells were transiently transfected with Empty Vector (EV) or myc-β-catenin (WT) followed by TRAIL-TZD treatment for 16 hrs. Western Blots were performed next with the indicated antibodies.

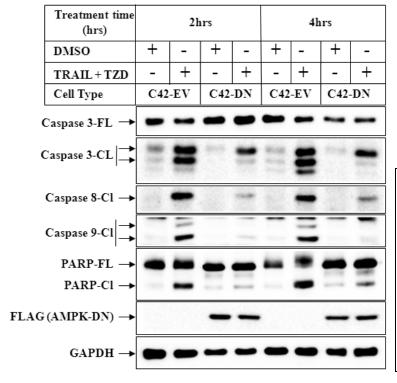*Effect of PPARγ knockdown on TRAIL-TZD-induced apoptosis:*
Since TZD is a ligand of PPARγ and to rule out any PPARγ-independent effects of TZD in our studies, we determined whether PPARγ was involved in mediating the effects. PPARγ expression

Cell Type	DU145							
siRNA	Control				PPARγ			
Treatment time	4hrs		8hrs		4hrs		8hrs	
DMSO	+	-	+	-	+	-	+	-
TRAIL (100ng/ml)	-	+	-	+	-	+	-	+
TZD (50µM)	-	+	-	+	-	+	-	+

was knocked down in the DU145 cells with PPARγ-siRNA followed by TRAIL-TZD treatment and Western Blot analysis. These showed (Fig 12) an optimal reduction of endogenous PPARγ expression with PPARγ-siRNA, which significantly attenuated caspase 3 cleavage, although the effects on upstream caspases (8 and 9) and PARP were modest.

Fig 12: Effect of PPARγ knockdown on TRAIL-TZD-induced apoptosis: DU145 cells were transiently transfected with control or PPARγ-siRNA for 72hrs followed by TRAIL-TZD treatment for 4 and 8hrs. The samples were analyzed by Western Blots with the antibodies indicated.

As described in our last annual report, we have determined a very novel pathway involving AMP-activated Kinase (AMPK) that participates in TRAIL-TZD-induced apoptosis and β-catenin modulation. The results of these are described below:
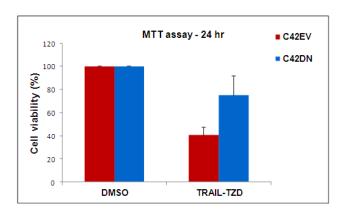
Treatment time (hrs)	2hrs				4hrs			
DMSO	+	-	+	-	+	-	+	-
TRAIL + TZD	-	+	-	+	-	+	-	+
Cell Type	C42-EV		C42-DN		C42-EV		C42-DN	

Effect of AMPK dominant negative mutant (AMPK-DN) on TRAIL-TZD-induced apoptosis:
Earlier reports have shown that AMP-activated Kinase (AMPK) activation can ameliorate TRAIL resistance and sensitize cells to TRAIL-induced apoptosis (19).

Fig 13: Effect of AMPK-Dominant negative (DN) mutant on TRAIL-TZD-induced apoptosis: C42-prostate cancer cells stably overexpressing Empty vector (C42-EV) or Flag-tagged AMPK-DN mutant (C42-DN) were treated with either DMSO or TRAIL-TZD combination for the indicated amounts of time. The samples were analyzed by Western Blots utilizing the antibodies indicated.

Since Troglitazone (TZD) used in our studies can also activate AMPK (20), we hypothesized that TRAIL-TZD-induced apoptosis might be mediated via AMPK pathway. To determine whether AMPK participates in this pathway, we utilized two different prostate cancer cells lines, one that expressed empty vector (C42-EV) and the other that expressed AMPK dominant negative mutant (C42-DN) as described previously (21). TRAIL-TZD studies designed with these two cell types showed a potent induction of apoptosis pathway following TRAIL-TZD addition in C42-EV cells (see c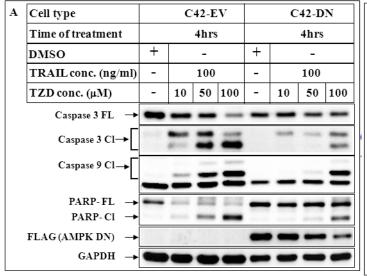leaved caspases and cleaved PARP), which was antagonized significantly in the C42-DN cells (Fig 13). These results suggested a potential involvement of AMPK in mediating TRAIL-TZD-induced apoptosis. To confirm that AMPK is involved in mediating this apoptosis pathway, MTT assays were also performed

Fig 14: Effect of AMPK-DN on Cell survival: C42 and C42-DN cells were treated as in Fig 13 followed by MTT assay. Percent cell viability was calculated considering the values of DMSO treatment as 100% for each cell type.

and the % survival of cells determined in both cell types. These data are shown in Fig 14, which also showed that AMPK-DN increased cell survival compared to EV cells when treated with TRAIL-TZD combination.

Effect of AMPK-DN on apoptosis with increasing concentrations of TRAIL or TZD: Once we observed that AMPK-DN antagonizes TRAIL-TZD-induced apoptosis (Fig 13), we designed studies to determine its effect on increasing concentrations of TRAIL or TZD in TRAIL-TZD
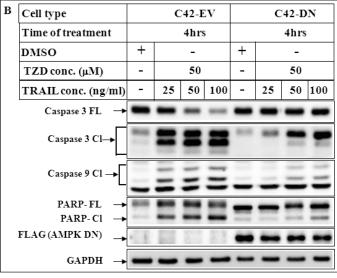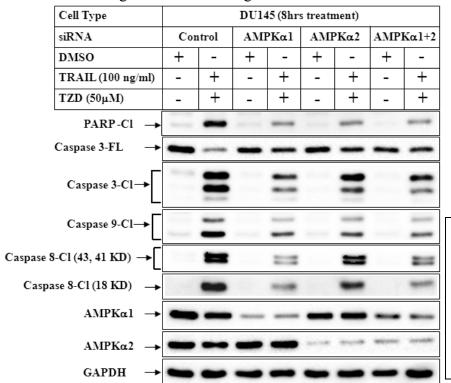

Fig 15: Effect of AMPK-DN on apoptosis with increasing concentrations of TRAIL and TZD: C42 and C42-DN cells were treated with either DMSO or 100ng/ml TRAIL and increasing concentrations of TZD **(A)** and DMSO or 50μM TZD and increasing concentrations of TRAIL **(B)**. Cells were harvested after 4 hours and samples were analyzed by Western Blots utilizing the antibodies indicated.

combination. These results (Figs 15 A&B) showed that AMPK-DN attenuated apoptosis even at higher concentrations of TRAIL (upto 100ng/ml) and TZD (upto 100μM).
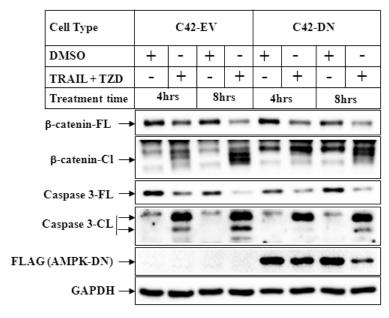
Effect of AMPK knockdown on TRAIL-TZD-induced apoptosis:
To determine whether endogenous AMPK was involved in mediating this apoptosis, endogenous AMPKα1 and α2 were knocked down in DU145 prostate cancer cells by their respective siRNAs. Fig 16 showed a significant knockdown of each isoform with the corresponding siRNAs. TRAIL-TZD studies performed following this knockdown showed an attenuation of apoptosis following knockdown of either AMPKα1 or α2, but it was predominantly mediated by AMPKα1. These results combined suggested that AMPK is involved in mediating TRAIL-TZD-induced apoptosis.

Cell Type	DU145 (8hrs treatment)							
siRNA	Control		AMPKα1		AMPKα2		AMPKα1+2	
DMSO	+	-	+	-	+	-	+	-
TRAIL (100 ng/ml)	-	+	-	+	-	+	-	+
TZD (50μM)	-	+	-	+	-	+	-	+

Fig 16: Effect of knockdown of AMPKα1 and α2 on TRAIL-TZD-induced apoptosis: DU145 cells were transiently transfected with control or AMPKα1 or α2 siRNA alone or in combination for 72hrs followed by TRAIL-TZD treatment for 8hrs. The samples were analyzed by Western Blots with the antibodies indicated.

Effect of AMPK-DN on TRAIL-TZD-induced modulation of β-catenin:
Once we confirmed that AMPK was involved in mediating the apoptotic response following TRAIL-TZD stimulation, we determined whether this also regulated β-catenin cleavage. This was based on our earlier results that showed that β-catenin cleavage following TRAIL-TZD stimulation

Cell Type	C42-EV				C42-DN			
DMSO	+	-	+	-	+	-	+	-
TRAIL + TZD	-	+	-	+	-	+	-	+
Treatment time	4hrs		8hrs		4hrs		8hrs	

Fig 17: Effect of AMPK-DN on TRAIL-TZD-induced β-catenin cleavage: C42 and C42-DN cells were treated with DMSO or TRAIL-TZD for the indicated amounts of time. The samples were analyzed by Western Blots with the antibodies indicated.

15

was mediated by caspases (Fig 3). These results shown in Fig 17 indicated that TRAIL-TZD-induced β-catenin cleavage was at least partially attenuated by AMPK-DN. Thus based on these studies it is conceivable that AMPK is involved in mediating TRAIL-TZD-induced caspase activation and apoptosis which then mediates the cleavage of β-catenin protein.
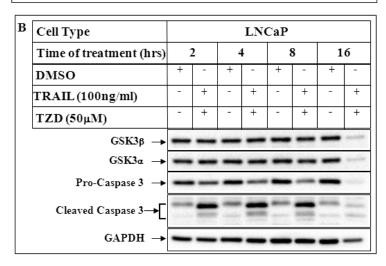

Task 2: To determine the role of GSK3β in potentiating drug-induced β-catenin cleavage and apoptosis (months 1-24)
Subaims 2(i)-(iii):
Studies in this aim were designed to determine whether TRAIL-TZD combination modulates GSK3β pathway and the effect of GSK3β inhibition towards drug-induced apoptosis in prostate cancer cells.

Effect of TRAIL-TZD stimulation on GSK3β expression and activation: To understand the role of GSK3β in mediating this apoptosis response, studies were performed first to see if TRAIL-TZD modulated GSK3β expression or activation and to determine its correlation with apoptosis. These were achieved by treating LNCaP (AR positive) and DU145 (AR negative) prostate cancer cells with TRAIL-TZD for different lengths of time and analyzing the effects on total GSK3β expression and GSK3β^{Ser9} phosphory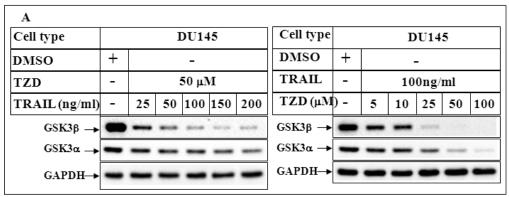lation (indicates inactivation of GSK3β). These results are shown in Fig 18, which indicated a time dependent decrease in total GSK3β and GSK3α expressions and an increase in GSK3β^{Ser9} phosphorylation following this combination treatment. In fact, the increase in GSK3β^{Ser9} seemed to correlate with Caspase 3 cleavage (or apoptosis) and the decrease in total GSK3β expression occurred later. This indicated that TRAIL-TZD can antagonize GSK3β pathway by two mechanisms, first via increasing the inhibitory phosphorylation at Ser9 and second via reducing the total expression. In addition, this antagonism is evident in various prostate cancer cells irrespective of the AR status.

Fig 18: Effect of TRAIL-TZD on GSK3 α and β pathways in prostate cancer cells: DU145 **(A)** and LNCaP **(B)** cells were treated with DMSO or a combination of TRAIL and TZD for the indicated amounts of time. The lysates were then analyzed by Western Blots with the antibodies indicated.

Effect of increasing concentrations of TRAIL and TZD on inhibiting total GSK3β expression: To determine the optimal concentration of TRAIL and TZD required for reducing GSK3β and GSK3α expressions maximally, Western Blot analyses were performed with DU145 and LNCaP cell extracts that were treated with increasing concentrations of either TZD or TRAIL. The results indicated that 50-100μM TZD when combined with 100ng/ml TRAIL is optimal for maximal reduction of both GSK3β and GSK3α expressions (Fig 19 A&B).
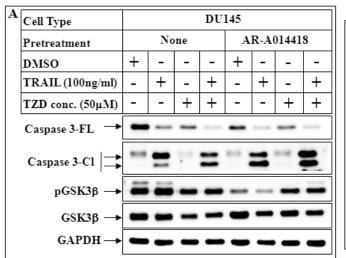
A

Cell type	DU145						Cell type	DU145					
DMSO	+	–					DMSO	+	–				
TZD	–	50 μM					TRAIL	–	100ng/ml				
TRAIL (ng/ml)	–	25	50	100	150	200	TZD (μM)	–	5	10	25	50	100

GSK3β →

GSK3α →

GAPDH→

B

Cell type	LNCaP								
Time of treatment	16hrs								
DMSO	+	–				–			
TZD conc. (μM)	–	50				10	25	50	100
TRAIL conc. (ng/ml)	–	25	50	100	150	100			

GSK3β →

GSK3α →

GAPDH→

Fig 19: Effect of increasing concentrations of TZD and TRAIL on prostate cancer cell apoptosis: **(A)** DU145 cells were treated with DMSO or a combination of 50μM TZD and increasing concentrations of TRAIL (25-200ng/ml) or 100ng/ml TRAIL and increasing concentrations of TZD (5-100μM). Cells were harvested after 24hours of treatment, followed by Western Blot analysis with the indicated antibodies. **(B)** LNCaP cells were treated as indicated and analyzed by Western Blots as in A.

Effect of GSK3β inhibition on TRAIL-TZD-induced apoptosis:
To determine whether GSK3β inhibition promotes apoptosis in prostate cancer cells, we first determined the effect of GSK3β inhibitor AR-A014418 on TRAIL-TZD-induced apoptosis in DU145 cells. These results shown in Fig 20A indicated that AR-A014418 can sensitize the cells to TRAIL-induced apoptosis even in the absence of TZD (compare lanes 2, 4 and 6). This suggested that AR-A014418-induced GSK3β inhibition promotes apoptosis in prostate cancer cells and is independent of the AR status. In additional experiments, DU145 cells were pretreated with a different inhibitor of GSK3β, BIO followed by TRAIL-TZD treatment which also showed that BIO can sensitize towards TRAIL-induced apoptosis (Fig 20B).
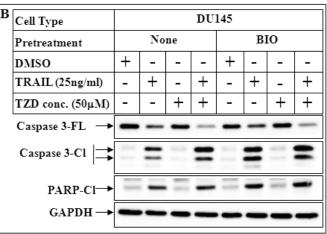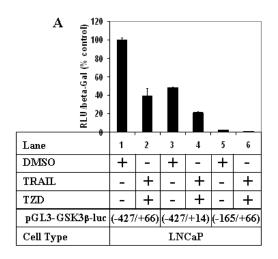

Fig 20: Effect of GSK3β inhibition on TRAIL-TZD-induced apoptosis in DU145 cells: DU145 cells were treated with the indicated concentrations of TRAIL or TZD alone or in combination in the presence or absence of a pretreatment with 20μM AR-A014418 (A) or 2μM BIO (B). Cells were harvested after 16hrs of treatment in A and after 8hrs of treatment in B and analyzed by Western Blots.

Mechanism of TRAIL-TZD-mediated suppression of total GSK3β expression:
To understand the mechanism by which TRAIL and TZD combination might be regulating total GSK3β expression, and whether this regulation is at the level of transcription, TRAIL-TZD studies were designed with three different GSK3β promoter luciferase reporters pGL3-GSK3β-luc (-427/+66, -427/+14 and -165/+66) as described earlier (22). These results showed that although the basal promoter activity was maximal with the -427/+66 bp construct, treatment with TRAIL and TZD combination produced a significant inhibition of promoter activity with all the constructs, thus suggesting that the-165/+66 bp construct contains the minimal responsive element (Fig 21 A&B). These also confirmed that TRAIL-TZD regulated total GSK3β at a transcriptional level. However, more detailed and mechanistic studies are needed to conclude whether this is exclusively transcriptional effect.
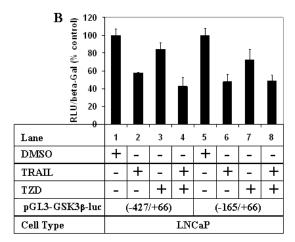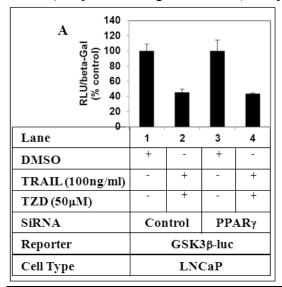
18

Fig 21: Effect of TRAIL-TZD on GSK3β promoter activity: LNCaP cells were transiently transfected with various GSK3β-promoter luciferase constructs along with β-Galactosidase (as control). 48 hours after transfection, they were treated with either DMSO or TRAIL-TZD combination (A) or with TRAIL, TZD and TRAIL-TZD combination (B) for 10 hours. Luciferase and β-Gal assays were performed next and represented as RLU/beta-Gal considering the DMSO control as 100%.

Effect of PPARγ knockdown on TRAIL-TZD-attenuated GSK3β expression:

We next determined whether PPARγ was involved in mediating the effects of TRAIL-TZD on inhibition of GSK3β expression by knocking down PPARγ expression by PPARγ-siRNA. These results showed that despite a significant reduction of endogenous PPARγ expression by PPARγ-siRNA, there was no effect on TRAIL-TZD-induced reduction of GSK3β expression (Fig 22B) and promoter activity (Fig 22A). These suggested that TRAIL-TZD-induced attenuation of GSK3β expression might be PPARγ independent.
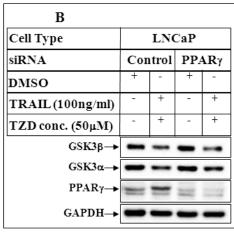

Fig 22: Effect of PPARγ knockdown on TRAIL-TZD-attenuated GSK3β expression: (A) LNCaP cells were transfected with control or PPARγ- siRNA along with GSK3β-promoter luciferase construct (-427/+66) followed by TRAIL-TZD treatment for 6hrs. Luciferase and β-Gal assays were performed as in Fig 21. **(B)** LNCaP cells were transfected with control or PPARγ- siRNA followed by TRAIL-TZD treatment for 16hrs and Western Blot analyses.

Task 3: Whether β-catenin mediates drug-induced apoptosis in prostate xenografts *in vivo*
(months 1-36)
Studies in this section are expected to address the role of TRAIL-TZD combination as well as β-catenin in mediating apoptosis *in vivo* utilizing a subcutaneous xenograft model. These were initially planned with SCID mice to be injected with LNCaP prostate cancer cells and following approval from Loyola IACUC and ACURO, the xenograft studies were initiated. As indicated in the past funding cycle, due to a very poor tumor take (only 1 out of 5) and a slow tumor growth (> 5 weeks to observe very small palpable tumor) in SCID mice, the initial animal protocol was amended to perform these xenograft studies in nude mice, which is reported to have better tumor take. However, despite the change of mouse strain, no palpable tumors were detected until 8 weeks after injection with LNCaP cells at which time mice were euthanized. This has created a setback in these xenograft studies that were initially planned under this task. Since then, I have specifically discussed this problem with some of the experts in prostate cancer research during American Association for Cancer Research (AACR) annual meeting of 2012. As suggested by these experts, LNCaP cells in general seems to have very poor tumor take in subcutaneous xenograft models and this can be overcome by using the LNCaP derivative cells LNCaP-C4-2, LNCaP-C4-2B. Accordingly, we have obtained the C42 and C42B cells and have utilized them *in vitro* to determine how they respond to TRAIL and TZD combination, as was initially planned. TRAIL-TZD studies performed with these cells showed a significant induction of apoptosis in both cell types following incubation with this combination as shown in Fig 23.
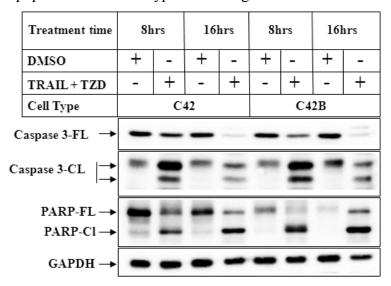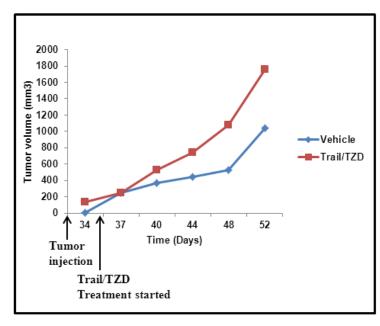

Treatment time	8hrs		16hrs		8hrs		16hrs	
DMSO	+	-	+	-	+	-	+	-
TRAIL + TZD	-	+	-	+	-	+	-	+
Cell Type	C42				C42B			

Caspase 3-FL →
Caspase 3-CL →→
PARP-FL →
PARP-Cl →
GAPDH →

Fig 23: Effect of TRAIL-TZD on the apoptosis of C42 and C42B prostate cancer cells: C42 and C42B cells were treated with DMSO or TRAIL-TZD as in Fig 2 and harvested at the indicated time points. Western Blot analyses were performed with the antibodies indicated.

Based on these results, we have modified our ACORP to include both of these cell types for our xenograft injection. After receiving approval from our institutional IACUC (dated Jan, 2013), and from ACURO (dated March, 2013), we have initiated these new xenograft studies with the C42 and C42B cells in nude mice. Our results showed that mice injected with C42 cells had a 60% tumor take (6 out of 10), while those injected with C42B didn't develop any tumors. Two of the 6 mice that developed tumors were euthanized as per our institutional tumor burden policy, since the tumors grew very fast. Of the remaining 4 mice with palpable tumors, two were treated with vehicle and two were treated with a combination of TRAIL and TZD for ~52 days and

tumor volumes were estimated. The results from these are shown in Fig 24, which indicated an increase in

Fig 24: Effect of TRAIL-TZD on C42 xenografts: Nude mice were injected with C42 cells and when palpable tumors were formed (~35 days), they received either vehicle or a combination of TRAIL (10mg/Kg/day) and TZD (50mg/Kg/day). Tumor measurements were taken by calipers and tumor volumes estimated upto 52 days.

tumor volume in vehicle-treated mice with time. Treatment with TRAIL and TZD combination, however, didn't effectively reduce tumor growth within this time period.

Key Research Accomplishments:

1. Identified the prostate cancer cell type that is maximally responsive to the combinatorial treatment with TRAIL and TZD. These also showed that the androgen dependent prostate cancer cells (LNCaP) are the most responsive to this drug combination.
2. Received information regarding the optimal concentration of TRAIL and TZD and the plating cell density at which apoptosis is maximal.
3. Determining a time dependent correlation between β-catenin cleavage and apoptosis induction in prostate cancer cells.
4. Determining a correlation of HDAC inhibitor-TRAIL-induced apoptosis and β-catenin cleavage.
5. Determining that β-catenin cleavage during apoptosis is mediated via activation of Caspases 3 and 8.
6. Determining the localization of the cleaved β-catenin fragment during TRAIL-TZD-induced apoptosis.
7. Characterization the status of interaction between cleaved β-catenin, TCF4, α-catenin and E-cadherin.
8. Creation of cleavage-resistant (D/A) myc-tagged β-catenin mutants and their characterization on TRAIL-TZD-induced apoptosis and β-catenin cleavage.
9. Mapping of additional cleavage sites on β-catenin following TRAIL-TZD treatment by Mass Spectrometry.
10. Determining the effect of β-catenin knockdown in increasing apoptotic sensitivity in AR-positive LNCaP cells following TRAIL-TZD stimulation.
11. Determining the effect of β-catenin overexpression on LNCaP cell apoptosis.
12. Determining the involvement of PPARγ in mediating TRAIL-TZD-induced apoptosis.
13. Determining the involvement of AMPK in mediating the apoptosis pathway following TRAIL-TZD treatment.
14. Determining the involvement of AMPK in mediating β-catenin cleavage following TRAIL-TZD treatment.

15. Determining the effect of TRAIL-TZD on GSK3β and GSK3α pathways in AR-positive and AR-negative prostate cancer cells.
16. Received information regarding the optimal concentration of TRAIL and TZD required for maximal effect on GSK3β pathway.
17. Determining the effect of various GSK3β inhibitors on increasing TRAIL sensitivity in prostate cancer cells.
18. Determining the effect of TRAIL-TZD on GSK3β transcription and the role of PPARγ.
19. Characterization of the LNCaP derivative cells C4-2 and C4-2B for TRAIL-TZD response *in vitro* so they can be utilized for *in vivo* xenograft studies.
20. Optimizing the xenograft injection in nude mice with C42 and C42B cells and tumor volume measurement following TRAIL-TZD treatment.

Reportable Outcomes:

We had an initial set-back in the overall studies, since two personnel have left the program during the course of the funding period. Since then new personnel has been hired and after an initial delay (covering the training period) we have made significant progress in the studies and are working towards publishing the manuscripts within this year. The following manuscripts are currently been prepared.

Manuscript:

1. Majumdar S, Thylur R, Rangasamy V, Rana A and **Rana B**. Modulation of GSK3beta during TRAIL-TZD-induced apoptosis in prostate cancer cells. *(Manuscript under preparation).*

2. Cheng YH, Majumdar S, and **Rana B**. Role of beta-catenin in mediating cell survival in TRAIL-TZD-induced apoptosis in prostate cancer cells. *(Manuscript under preparation).*

3. Santha S, Majumdar S, Rana A and Rana B. Involvement of AMPK in mediating TRAIL-TZD-induced beta-catenin cleavage and apoptosis. *(Manuscript under preparation).*

Abstracts:

- Thorenoor N, Thylur R, Rana A and **Rana B**. Role of GSK3β in modulating TRAIL-induced apoptosis in prostate cancer cells. Presented at AACR Annual Meeting, March 31-April 4, 2012, Chicago, IL (see attached abstract under appendix).

- Thylur RP, Thorenoor N, Rangasamy V, Rana A and **Rana B**. Role of ß-catenin in prostate cancer cell apoptosis. Presented at AACR Annual Meeting, March 31-April 4, 2012, Chicago, IL (see attached abstract under appendix).

- Majumdar S, Santha S, Rana A and **Rana B**. Regulation of GSK3β axis by combination treatment with TRAIL and Troglitazone in cancer cells. Presented at AACR Annual Meeting, April 5-9, 2014, San Diego, CA (see attached abstract under appendix).

- Santha S, Majumdar S, Viswakarma N, Rana A and **Rana B**. TRAIL-TZD combinatorial treatment induces apoptosis in prostate cancer cells through modulation of AMPK signaling pathway. Presented at AACR Annual Meeting, April 5-9, 2014, San Diego, CA (see attached abstract under appendix).

Conclusions:

In conclusion, we have demonstrated utilizing both androgen dependent and independent prostate cancer cells that the androgen dependent cell types (LNCaP cells mostly) respond to this TRAIL-TZD combination treatment via inducing apoptosis. This pathway of apoptosis is always associated with an increased cleavage of β-catenin, and inhibition of β-catenin expression. This cleavage of β-catenin is also observed in other pathways of apoptosis, including treatment with a combination of TRAIL and HDAC inhibitors and is mediated via activation of Caspases. Differential fractionation studies indicated that, although the full-length β-catenin protein is localized on the membrane, the cleaved fragment is localized exclusively in the soluble fraction along with cleaved Caspase 3. In addition, this cleavage significantly reduces interaction of β-catenin with its transcriptional binding partner TCF4, whereas it still retains the interaction with E-cadherin and α-catenin, which might be critical in the induction of apoptosis as well as β-catenin cleavage. In addition, studies with β-catenin-siRNA revealed novel information that suggests that in prostate cancer cells β-catenin might be promoting the apoptotic resistance. More molecular studies are planned to confirm this, which will reveal important downstream mediators of this pathway. Utilizing two different GSK3β inhibitors (AR-A014418 and BIO), we have shown that GSK3β inhibition promotes TRAIL sensitivity independent of AR expression. In addition, TRAIL-TZD antagonizes GSK3β and GSK3α pathways in these cells via up-regulating pGSK3β^{Ser9} levels (indicating inhibition), and down-regulating total expression of both GSK3α and GSK3β. Since Troglitazone used in the combination studies can activate AMPK, we also investigated whether AMPK was involved in mediating this apoptosis by overexpressing AMPK-dominant negative (DN) mutant. These revealed a significant inhibition of the apoptosis pathway in the presence of AMPK-DN, suggesting involvement of AMPK. Studies are currently underway to elucidate the detailed pathway. We have also obtained and characterized the C42 and C42B cells, both of which respond potently to TRAIL-TZD-induced apoptosis. Xenograft studies showed C42 cells can form subcutaneous xenografts in nude mice, although TRAIL-TZD combination was unable to reduce prostate tumor progression.

References:

1. Tindall, D. J., and Scardino, P. T. (1999) Defeating prostate cancer: crucial directions for research--excerpt from the report of the Prostate Cancer Progress Review Group. *Prostate* 38, 166-171
2. Hsing, A. W., Tsao, L., and Devesa, S. S. (2000) International trends and patterns of prostate cancer incidence and mortality. *Int J Cancer* 85, 60-67
3. Bienz, M., and Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. *Cell* 103, 311-320
4. Gounari, F., Signoretti, S., Bronson, R., Klein, L., Sellers, W. R., Kum, J., Siermann, A., Taketo, M. M., von Boehmer, H., and Khazaie, K. (2002) Stabilization of beta-catenin induces lesions reminiscent of prostatic intraepithelial neoplasia, but terminal squamous transdifferentiation of other secretory epithelia. *Oncogene* 21, 4099-4107
5. Bierie, B., Nozawa, M., Renou, J. P., Shillingford, J. M., Morgan, F., Oka, T., Taketo, M. M., Cardiff, R. D., Miyoshi, K., Wagner, K. U., Robinson, G. W., and Hennighausen, L. (2003) Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation. *Oncogene* 22, 3875-3887
6. Chesire, D. R., Ewing, C. M., Gage, W. R., and Isaacs, W. B. (2002) In vitro evidence for complex modes of nuclear beta-catenin signaling during prostate growth and tumorigenesis. *Oncogene* 21, 2679-2694
7. Chesire, D. R., Ewing, C. M., Sauvageot, J., Bova, G. S., and Isaacs, W. B. (2000) Detection and analysis of beta-catenin mutations in prostate cancer. *Prostate* 45, 323-334
8. Voeller, H. J., Truica, C. I., and Gelmann, E. P. (1998) Beta-catenin mutations in human prostate cancer. *Cancer Res* 58, 2520-2523
9. Yang, F., Li, X., Sharma, M., Sasaki, C. Y., Longo, D. L., Lim, B., and Sun, Z. (2002) Linking beta-catenin to androgen-signaling pathway. *J Biol Chem* 277, 11336-11344
10. Mulholland, D. J., Cheng, H., Reid, K., Rennie, P. S., and Nelson, C. C. (2002) The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. *J Biol Chem* 277, 17933-17943
11. Truica, C. I., Byers, S., and Gelmann, E. P. (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Res* 60, 4709-4713
12. Dihlmann, S., Kloor, M., Fallsehr, C., and von Knebel Doeberitz, M. (2005) Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. *Carcinogenesis* 26, 1503-1512
13. Xie, H., Huang, Z., Sadim, M. S., and Sun, Z. (2005) Stabilized beta-catenin extends thymocyte survival by up-regulating Bcl-xL. *J Immunol* 175, 7981-7988
14. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 5, 157-163

15. Van Ophoven, A., Ng, C. P., Patel, B., Bonavida, B., and Belldegrun, A. (1999) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for treatment of prostate cancer: first results and review of the literature. *Prostate Cancer Prostatic Dis* 2, 227-233

16. Lu, M., Kwan, T., Yu, C., Chen, F., Freedman, B., Schafer, J. M., Lee, E. J., Jameson, J. L., Jordan, V. C., and Cryns, V. L. (2005) Peroxisome proliferator-activated receptor gamma agonists promote TRAIL-induced apoptosis by reducing survivin levels via cyclin D3 repression and cell cycle arrest. *J Biol Chem* 280, 6742-6751

17. Sharma, C., Pradeep, A., Wong, L., Rana, A., and Rana, B. (2004) Peroxisome proliferator-activated receptor gamma activation can regulate beta-catenin levels via a proteasome-mediated and adenomatous polyposis coli-independent pathway. *J Biol Chem* 279, 35583-35594

18. Thylur, R. P., Senthivinayagam, S., Campbell, E. M., Rangasamy, V., Thorenoor, N., Sondarva, G., Mehrotra, S., Mishra, P., Zook, E., Le, P. T., Rana, A., and Rana, B. (2011) Mixed lineage kinase 3 modulates beta-catenin signaling in cancer cells. *The Journal of biological chemistry* 286, 37470-37482

19. Pradelli, L. A., Beneteau, M., Chauvin, C., Jacquin, M. A., Marchetti, S., Munoz-Pinedo, C., Auberger, P., Pende, M., and Ricci, J. E. (2010) Glycolysis inhibition sensitizes tumor cells to death receptors-induced apoptosis by AMP kinase activation leading to Mcl-1 block in translation. *Oncogene* 29, 1641-1652

20. LeBrasseur, N. K., Kelly, M., Tsao, T. S., Farmer, S. R., Saha, A. K., Ruderman, N. B., and Tomas, E. (2006) Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *American journal of physiology. Endocrinology and metabolism* 291, E175-181

21. Zhou, J., Huang, W., Tao, R., Ibaragi, S., Lan, F., Ido, Y., Wu, X., Alekseyev, Y. O., Lenburg, M. E., Hu, G. F., and Luo, Z. (2009) Inactivation of AMPK alters gene expression and promotes growth of prostate cancer cells. *Oncogene* 28, 1993-2002

22. Zhang, J. S., Koenig, A., Harrison, A., Ugolkov, A. V., Fernandez-Zapico, M. E., Couch, F. J., and Billadeau, D. D. (2011) Mutant K-Ras increases GSK-3beta gene expression via an ETS-p300 transcriptional complex in pancreatic cancer. *Oncogene* 30, 3705-3715

Appendices:

1. See Loyola IACUC approval letter (3 year renewal) and ACURO approval letter on the next page.
2. See 4 abstracts generated from these studies that were presented at the AACR annual meeting in 2012 and 2014.

January 25, 2013

U.S. Army Medical Research and Materiel Command
Animal Care and Use Review Office
ATTN: MCMR-RPA
504 Scott Street
Fort Detrick, MD 21702-5012
Email: acuro@amedd.army.mil

RE: IACUC APPROVAL
    PI: DR. B. RANA; LU#201974 (2.04) Mouse

To Whom It May Concern,

The Loyola University Chicago, Stritch School of Medicine's Institutional Animal Care and
Use Committee (IACUC) approved the ACORP; DR. B. RANA; LU#201974 (2.04) Mouse
(Beta catenin in prostate cancer apoptosis) on January 16, 2013. The protocol has a full board
approval until January 16, 2016.

Loyola University Chicago, Stritch School of Medicine has an Animal Assurance on file with
the Public Health Service under #A3117-01 approved through 02/28/2014, a fully AAALAC
International accredited institution (certification dated 11/10/2010), and USDA registered
/licensed institution under #33-R-0024 through 08/24/2014.

If you have any questions or require additional information, please feel free to contact the
IACUC Chair, Dr. Jawed Fareed via the Committee Director, telephone (708) 216
4288; Fax (708) 216-9399.

Sincerely,

Mr. Jamie Caldwell, MBA
Director
Office of Research Services
for the Health Sciences
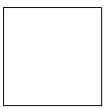Loyola University Chicago, Health Sciences Division
Health Sciences Campus
Bldg 120 Suite 400
2160 South First Avenue
Maywood, IL 60153

cc: LU/IACUC File: DR. B. RANA; LU#201974 (2.04) Mouse

March 01, 2013

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC093099, Award Number W81XWH-10-1-0195 entitled, "Beta Catenin in Prostate Cancer Apoptosis"

Principal Investigator Basabi Rana
Loyola University Medical Center
Chicago, IL

Dear Dr. Rana:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, the **rewrite** of protocol PC093099 entitled, "Beta Catenin in Prostate Cancer Apoptosis," IACUC protocol number 201974 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the Loyola University Chicago, Stritch School of Medicine IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding

    a. any serious or continuing noncompliance with the PHS Policy;
    b. any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
    c. any suspension of this activity by the IACUC

- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program
- AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

For

James Sheets, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
 Review Office

Copies Furnished:
Mr. Ayi Ayayi, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Nrusingha Mishra/MCMR-PLF
Dr. Basabi Rana, Loyola University Chicago, Stritch School of Medicine
Dr. Charlene J Repique, Congressionally Directed Medical Research Program (CDMRP)
Dr. Jawed Fareed, Loyola University Medical Center
Mr. Jamie Caldwell, Loyola University Chicago, Stritch School of Medicine

Role of GSK3β in modulating TRAIL-induced apoptosis in prostate cancer cells

(AACR Abstract Number: 238)

Nithyananda Thorenoor[1], Ramesh Thylur[1], Ajay Rana[2, 3] and Basabi Rana[1, 3,]

[1]Department of Medicine, Division of Gastroenterology, Hepatology & Nutrition , Loyola University Chicago, Maywood, IL 60153
[2]Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago, Maywood, IL 60153
[3]Hines VA Medical Center, Hines, IL 60141

Prostate cancer is the most commonly diagnosed malignancy and second leading cause of cancer death in males. Surgical excision and hormonal therapy are the first-line of treatments, which are not effective at later-stages. Designing efficient non-invasive therapeutic strategies that can target prostate cancer cells towards apoptosis is critically important, thus making the apoptotic signaling pathways as attractive targets for cancer therapy. The ability of cancer cells to evade apoptosis is a hallmark of oncogenesis, and a major setback in current therapy. TNF-related apoptosis inducing ligand (TRAIL) has gained much importance recently due to its ability to preferentially induce cell death in malignant and transformed cells but not in normal cells. However, since many tumor cells develop resistance to TRAIL, recent approaches are focused on developing combinatorial therapeutic regimens that can enhance TRAIL sensitivity. There are multiple reasons behind TRAIL resistance, and recent studies (including ours) indicate that, Glycogen Synthase Kinase 3β (GSK3β) might be a key player in mediating this. GSK3β, a multifunctional serine/threonine kinase regulates diverse physiological processes, depending on its substrates, and its kinase activity seems to be critical for various cancer cells. In our earlier studies, combinatorial treatment with Troglitazone (TZD), a synthetic ligand for peroxisome proliferator-activator receptor gamma and TRAIL induced significant apoptosis in TRAIL-resistant cancer cells. More in-depth analysis of the signaling pathways promoting TRAIL resistance indicated that pretreatment of resistant prostate cancer cells with a pharmacological inhibitor of GSK3β (AR-A014418) ameliorates TRAIL resistance and synergizes with TZD to induce potent apoptosis. In addition, small interference RNA (siRNA)-mediated knockdown of GSK3β expression promoted TRAIL-TZD-induced apoptosis, whereas ectopic expression of GSK3β antagonized this. Further studies indicated a TRAIL-TZD-induced decrease in total GSK3β expression and increase in GSK3β^{Ser9} phosphorylation (inactivation) in apoptotic cells.

Pre-treatment with caspase inhibitors protected the cells from apoptosis, but was unable to reverse the effects of TRAIL-TZD on GSK3β, suggesting these to be caspase independent. Pretreatment with protein synthesis inhibitor Cycloheximide abolished the inhibitory effects of TRAIL-TZD on total GSK3β, suggesting this to be a transcriptional event. In fact, luciferase assays indicated a significant reduction of GSK3β promoter activity following this combination treatment. The results from the present study show that, targeting GSK3β might be an effective mechanism of increasing TRAIL sensitivity, and understanding the detailed mechanism by which TRAIL-TZD combination antagonizes GSK3β pathway might provide novel insight to improve our understanding of TRAIL resistance.

Role of ß-catenin in prostate cancer cell apoptosis
(AACR Abstract Number: 251)

Ramesh P Thylur[1], Nithyananda Thorenoor[1], Velusamy Rangasamy[2], Ajay Rana[2, 3] and Basabi Rana[1, 2, 3]

[1]Department of Medicine, Division of Gastroenterology, Hepatology & Nutrition, Loyola University Chicago, Maywood, IL 60153

[2]Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago, Maywood, IL 60153

[3]Hines VA Medical Center, Hines, IL 60141

Introduction: Evasion of apoptosis is a hallmark of cancer cells, and apoptotic signaling pathways are attractive targets for cancer therapy. TNF-related apoptosis inducing ligand (TRAIL) has gained much importance recently due to its ability to preferentially induce apoptosis in transformed cells and not in normal cells. Since many tumor cells develop TRAIL resistance, developing combinatorial treatment strategies to enhance TRAIL sensitivity is a major challenge of current cancer research. The reasons behind TRAIL resistance are multiple, and might be linked with aberrant activation of various oncogenic pathways. ß-catenin is a well established oncogene being overexpressed in various cancers and might be involved in TRAIL resistance. Normally, it functions as a component of both the adherens junction complex and the Wnt/wingless signaling pathway. Our earlier studies have shown that treatment of cancer cells with Troglitazone (TZD), a synthetic ligand for Peroxisome Proliferator-activator receptor gamma can reduce cell proliferation and ß-catenin expression. When combined with TZD, TRAIL was capable of inducing potent apoptosis in various TRAIL-resistant cancer cells. Aims: The present study was designed to determine the involvement of ß-catenin during TRAIL-TZD-induced apoptosis of prostate cancer cells. Experimental procedures: Different prostate cancer cell lines (LNCaP, 22RV1, PC-3, DU145) were used to determine the effect of TRAIL-TZD on apoptosis and ß-catenin pathway, utilizing Western Blots, Immunoprecipitation, and luciferase assays. Results: Treatment with a combination of TRAIL and TZD resulted in significant apoptosis (PARP, caspase-3 cleavage) and ß-catenin cleavage in LNCaP but not in PC-3 and DU145 cells, and 22RV1 showed partial PARP cleavage. TRAIL-TZD-induced ß-catenin cleavage was antagonized by caspase inhibitors, suggesting this to be downstream of caspase activation. Luciferase assays with ß-catenin/TCF-responsive reporter indicated a significant reduction of luciferase activity with this treatment. Interestingly the cleaved ß-catenin fragment retained strong interaction with E-Cadherin, as indicated by immunoprecipitation studies. Detergent fractionation of the cell extracts treated with the drug combination revealed that ß-catenin cleavage was enriched in the cytosolic fraction. To understand any participation of HDACs, TRAIL treatment was performed in combination with various HDAC inhibitors. These results showed a strong increase in apoptosis and ß-catenin cleavage with TRAIL and valproic acid treatment, suggesting an involvement of HDACs in this apoptotic resistance. Conclusion: These results show that targeting ß-catenin signaling pathway might be effective means of increasing TRAIL sensitivity in prostate cancer, and combining TRAIL with TZD or valproic acid might be a promising treatment regimen for drug-resistant cells, especially those with aberrant ß-catenin expression.

TRAIL-TZD combinatorial treatment induces apoptosis in prostate cancer cells through modulation of AMPK Signaling pathway

Sreevidya Santha[1], Sunipa Majumdar[1], Navin Viswakarma[2], Ajay Rana[2, 3] and Basabi Rana[1, 2, 3]

[1]Department of Medicine, Division of Gastroenterology & Nutrition, Loyola University Chicago, Maywood, IL 60153

[2]Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago, Maywood, IL 60153

[3]Hines VA Medical Center, Hines, IL 60141

Prostate cancer is the second most frequently diagnosed cancer of men and the fifth most common cancer overall. Treatment options for localized disease include surgery, radiation therapy, and hormonal therapy, but they are not effective in the advanced stages of the disease. Most of the anticancer drugs in current use primarily act by inducing apoptosis in target cells. So, the identification of novel targets for drug-induced apoptosis will be useful for treating resistant forms. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapy that preferentially induces apoptosis in cancer cells. However, many neoplasms are resistant to TRAIL by mechanisms that are poorly understood. Our previous studies indicated that combinatorial treatment with Troglitazone (TZD), a synthetic ligand for peroxisome proliferator-activator receptor gamma and TRAIL can induce significant apoptosis in TRAIL-resistant cancer cells. In this study we determined AMP-activated protein kinase (AMPK) as a potential target for TRAIL-TZD-induced apoptosis in prostate cancer cells. AMPK is a family of serine/threonine protein kinase and is highly conserved from yeast to human. It consists of three subunits: a catalytic α subunit and regulatory β and γ subunits. AMPK is a well-accepted target for the treatment of metabolic syndrome and Type 2 diabetes. We used C42-DN (stably overexpressing AMPK α1-dominant negative) and C42-EV (empty vector) prostate cancer cell lines to study differences in TRAIL-TZD-induced apoptosis. Our studies showed a dose dependent increase in TRAIL-induced apoptosis with increasing concentrations of TZD (upto 100μM), which was significantly higher in C42EV cells compared with C42-DN cells. The peak of apoptosis was around 4h following treatment. Similarly, using MTT assay, we observed a dramatic reduction in cell viability in the C42-EV compared to C42-DN cells when treated with same

concentrations of TRAIL-TZD, thus suggesting that C42DN cells were more resistant to this treatment. In addition, time course studies with C42EV cells showed an increase in $pAMPK^{T172}$ levels (indicating AMPK activation) and $pACC^{S79}$ (downstream target of AMPK) levels coinciding with the time of apoptosis. Small interference RNA (siRNA)-mediated knockdown of endogenous AMPK α1 expression showed a reduction of apoptosis in DU145 and LNCaP cells. Studies are currently underway to determine whether knocking down both AMPK α1 and α2 produces a more complete inhibition of apoptosis. These studies suggest that apoptosis induction by TRAIL-TZD combination is mediated via AMPK in prostate cancer cells.

Regulation of GSK3β axis by combination treatment with TRAIL and Troglitazone in cancer cells

Sunipa Majumdar[1], Sreevidya Santha[1], Ajay Rana[2, 3] and Basabi Rana[1, 3,]

[1]Department of Medicine, Division of Gastroenterology & Nutrition, Loyola University Chicago, Maywood, IL 60153
[2]Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago, Maywood, IL 60153
[3]Hines VA Medical Center, Hines, IL 60141

Prostate cancer is estimated to account for 29% of all new cancers and is the second leading cause of cancer-related death in men in the United States. Hormonal therapy is the only treatment for advanced forms, but androgen-independence eventually develops in most patients. Developing new treatment strategies are urgently needed, which needs a deeper molecular understanding of the events that lead to tumor progression. TNF-related apoptosis inducing ligand (TRAIL) has gained much importance recently due to its ability to preferentially induce cell death in malignant and transformed cells. However, since many tumor cells develop resistance to TRAIL, recent approaches are focused on developing combinatorial therapeutic regimens that can enhance TRAIL sensitivity. There are multiple reasons behind TRAIL resistance, and recent studies (including ours) indicate that, Glycogen Synthase Kinase 3β (GSK3β) might be a key player in mediating this. Thus pathways that can antagonize GSK3β axis are important targets for cancer drug development. GSK3β is a serine/threonine kinase, initially identified as a critical mediator of glycogen metabolism and insulin signaling and is now well accepted to regulate various cellular processes including cell survival. In our earlier studies, combinatorial treatment with Troglitazone (TZD), a synthetic ligand for peroxisome proliferator-activator receptor gamma (PPARγ) and TRAIL induced significant apoptosis in TRAIL-resistant cancer cells. Utilizing this combination, we also observed an increase in GSK3β^{Ser9} phosphorylation that preceded the onset of apoptosis. At a later time, however, TRAIL-TZD combination produced a dramatic reduction of total GSK3β levels, suggesting that GSK3β is being targeted by multiple pathways following this treatment. Similar results were observed in various prostate as well as pancreatic cancer cells, indicating this to be a generalized event. Interestingly, pretreatment of Bx-PC3 pancreatic cancer cells (with a shorter half-life of GSK3β) with protein synthesis inhibitor Cycloheximide (CHX) significantly reduced TRAIL-TZD-induced inhibition

of total GSK3β expression, suggesting mostly a transcriptional regulation. Luciferase assays carried out in both pancreatic and prostate cancer cells indicated dramatic reduction of GSK3β promoter activity with TRAIL-TZD. Knockdown of endogenous PPARγ expression by PPARγ-small interference RNA (siRNA) resulted in a reduction of PPARγ levels with siRNA transfection, which was unable to antagonize TRAIL-TZD-induced reduction of GSK3β transcription or protein expression. Since the mechanisms that regulate GSK3β expression in cancer cells are largely unknown, these studies indicate a novel strategy of targeting GSK3β, elucidation of which might provide newer insights to improve our understanding of TRAIL resistance.